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(54) Title: SYNERGISM OF GOS AND POLYFRUCTOSE

(57) Abstract: The present invention relates to the filed of prebiotics. Provided are uses for compositions comprising synergistically effective amounts of polyfructose and galactooligosaccharides (GOS).

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**Synergism of GOS and polyfructose****Field of the invention**

The present invention relates to the field of human health and nutrition. It provides  
5 novel synergistic mixtures of prebiotic carbohydrates, especially mixtures of galacto-  
oligosaccharides (GOS, for example TOS) and polyfructose (for example inulin), as  
well as nutritional compositions comprising these. The nutritional compositions have  
beneficial effects when fed to bottle fed or partially bottle fed infants and also have  
health improving effects when ingested by adults having intestinal problems, such as  
10 Inflammatory Bowel Disease (IBD) or Irritable Bowel Syndrome (IBS).

**Background of the invention**

The microflora of the human large intestine (typically divided into caecum, colon and  
rectum) plays a crucial role in both human nutrition and health. The bacterial  
15 composition is influenced and can be modulated by dietary intake. Carbohydrates  
which have passed through the stomach and small intestine are metabolised by the  
bacteria and as a major end-product of metabolism short-chain fatty acids (SCFA), such  
as acetate, propionate, butyrate and valerate, are formed, which are subsequently  
released into the blood. Other end products of bacterial fermentation include for  
20 example lactate and succinate. The total amounts and compositions (relative amounts)  
of these end products in turn have a profound effect on bacterial growth, pH, exclusion  
of pathogenic species, etc. A method to beneficially influence the microbial flora and  
human health is the administration of prebiotics. "Prebiotics" were defined as "non-  
digestible food ingredients that beneficially affect the host by selectively stimulating  
25 the growth and/or activity of one or a limited number of bacteria in the colon, and thus  
improve the hosts health (Gibson and Roberfroid 1995, J. Nutr. 125, 1401-1412). The  
criteria which a compound must fulfil in order to be classified as a prebiotic are: 1) it  
must not be hydrolysed or absorbed in the upper part of the gastrointestinal tract  
(stomach, small intestine), 2) it must be selectively fermented by one or more  
30 potentially beneficial bacteria in the colon, 3) it must alter the colonic microbiota  
towards a healthier composition and 4) it must preferably induce effects which are  
beneficial to the health of the subject. Commonly used prebiotics are so-called non-  
digestible carbohydrates (or "soluble dietary fibres"), which pass undigested through

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the upper part of the gastrointestinal tract into the large intestine. These include for example fructooligosaccharides (FOS), oligofructose, inulin and transgalactooligosaccharides (TOS). It should be noted that in the literature, there is often inconsistency between the use of various terms, such as fructooligosaccharide, inulin, oligofructose and inulo-oligosaccharides, and the same term may be used for different compounds or compositions.

Well known beneficial bacteria, which are stimulated by the uptake of prebiotics, are lactic acid bacteria, such as *Lactobacilli* and *Bifidobacteria*, and health benefits have been ascribed to be due to this stimulatory effect. For example, beneficial effects of inulin and inulin-type fructans, such as oligofructose, on intestinal function have been described (Jenkins *et al.* 1999, J. Nutrition 129, 1431S-1433S) and this effect is thought to be due to a bifidogenic effect, which refers to a selective growth stimulating effect on total bifidobacteria, measured either *in vivo* through bifidobacterial counts of the faeces or *in vitro* (see e.g. Roberfroid, Am J Clin Nutr 2001, 73(suppl), 406S-409S).

Human milk appears also to have a bifidogenic effect, as the dominant bacteria which become established in breast-fed infants are bifidobacteria. In contrast, bacterial colonisation of milk formula fed infants is not dominated by bifidobacteria and is more diverse in bacterial species (Harmsen *et al.* 2000, J. Pediatr. Gastroenterol. Nutr. 30, 61-67). It is thought that oligosaccharides found in the human milk are responsible for the bifidogenic or prebiotic effect and efforts have been made to modify infant formula in such a way that it resembles human milk as closely as possible and especially that it has the same or a very similar prebiotic effect as human milk. This has been done by adding prebiotics to infant milk formula (Boehm *et al.* Acta Paediatr. Suppl. 2003, 441, 64-67 and Moro *et al.* 2002, J Pediatr Gastroenterol Nutr 34, 291-295). For example, supplementation of bovine milk formula with an oligosaccharide mixture comprising trans-galactooligosaccharides (TOS) and inulinHP has been described to increase the faecal count of bifidobacteria in bottle fed infants (Boehm *et al.* 2002, Arch Dis Child 86, F178-F181).

Also, supplementation of infant milk formula with TOS and inulin has been described to have a bifidogenic effect, and to decrease faecal pH (Boehm *et al.* 2003 supra; Moro

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*et al.* 2003, *Acta Paediatr. Suppl.* 441:77-79; Marini *et al.* 2003, *Acta Paediatr. Suppl.* 441:80-81; Boehm *et al.* 2002, *Arch. Dis. Child Fetal. Neonata. Ed.* 86:F178-F181; Moro *et al.* 2002, *J. Pediatr. Gastroenterol. Nutr.* 34:291-295; Schmelzle *et al.* 2003, *J. Pediatr. Gastroenterol. Nutr.* 36:343-51).

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Upon fermentation of prebiotics by lactic acid bacteria organic acids are produced and the pH is lowered. *Lactobacilli* produce either lactate or lactate and acetate (a Short Chain Fatty Acid; SCFA). The lactate can be in the L- or D- form. *Bifidobacteria*, on the other hand, produce L-lactate and acetate, but no D-lactate. *Bifidobacteria* (and other lactic acid bacteria) usually do not lead to the production of gases, such as H<sub>2</sub> and CH<sub>4</sub>. They also do not produce other SCFA, such as propionate, butyrate, isobutyrate, valerate and isovalerate. The presence of SCFA, such as isobutyrate and isovalerate, are indicative of the fermentation of carbohydrates by other bacterial species, such as *Clostridia* and *Bacteroides* or *Enterobacteriaceae*, or are indicative for the fermentation of proteins (of which the *Bifidobacteria* have a poor capability). Also, other intestinal bacteria are capable of producing acetate or lactate, such as *Propionibacteria*, *Enterococci* and *Pediococci*.

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Previously it has been reported that the intake of certain prebiotic carbohydrates increase the (relative) amounts of *Bifidobacteria* and/or *Lactobacilli*. Concomitantly an increased formation of SCFA and decrease of pH has been observed. But also the formation of gases, which results in unwanted symptoms such as flatulence and abdominal pain, has been reported when introducing prebiotics, such as inulin or GOS, into the diet. Furthermore, not only acetate but also increases in, for example, butyrate have been reported, which is undesirable. A range of undesirable effects have therefore been described, resulting from the consumption of nutrition supplemented with certain prebiotics.

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In the colon and faeces of breast fed infants the predominant SCFA found is acetate. Furthermore high concentrations of lactate are found. These (relative) amounts are higher than those observed in adults (where concentrations of lactate are generally negligible) or in standard milk formula fed infants. Subsequently, concentrations of propionate and especially butyrate are much lower in the colon and faeces of breast fed

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infants than in adults and even lower than in infants fed with standard infant milk formula. The pH of the faeces is lowest in breast fed children. As mentioned above, it is desirable to provide nutrient compositions, especially milk formula compositions, which, when consumed, result in an intestinal microflora closely resembling that of breast fed infants.

Many health effects of SCFA and lactate and a low colon pH have been described. The lowered pH and the presence of organic acids have been described to have an anti-pathogenic effect, and provide an advantage to acid tolerant bacteria such as the lactic acid bacteria (including *Bifidobacteria*). Also effects of SCFA on the intestinal wall have been described. SCFA are an energy source of colonocytes and thereby aid to the intestinal barrier integrity. SCFA are also involved in effects on peristalsis, bile acid metabolism, water absorption and cell differentiation (see e.g. EP1105002). SCFA are known to stimulate the production of mucus and are involved in mineral absorption and mucus production.

#### Detailed description of the invention

The present inventors surprisingly found that GOS (especially TOS) and polyfructose (especially inulin), when administered together, provide a number of beneficial effects which cannot be explained by an increase in the number of intestinal bifidobacteria and which are, therefore, not due to the bifidogenic effect described for TOS or inulinHP in the prior art (e.g. in Boehm *et al.* 2002, *supra*). In particular, it was unexpectedly found that, following administration of GOS and polyfructose mixtures, there was an increase in the total amounts of SCFA formed, an increase in the relative amounts of acetate and lactate and a decrease in faecal pH of infants, whereby these effects were not correlated with an increased (relative) amount of *Bifidobacteria*. Beside a beneficial SCFA amount and profile and an increased amount of lactate, a decrease in gas formation was observed. Further, lactate itself was found to have a hitherto unknown beneficial effect on the colon, as it was found to increase the secretion of prostaglandin E1 and prostaglandin E2. This effect was previously only reported for SCFA, especially acetate (Willemsen *et al.* 2003, Gut 52, 1442-1447). Compositions comprising lactate, and compositions comprising prebiotics which stimulate lactate production, may therefore be used to stimulate mucin production and support the mucosal barrier integrity

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(mucoprotection). Lactate was further found to decrease spontaneous contractions and tension in colonic muscles, resulting in relief of cramp and pain.

5 The prebiotic compositions are therefore novel, in that they do not, or do not significantly, increase the number of *Bifidobacteria*, while they do result in an increase in total SCFA amounts, beneficial changes in SCFA profile (increases in acetate, decreases in butyrate and propionate), increase in lactic acid (and resulting beneficial contraction and tension reducing effects and muco-stimulatory effect), a decrease in faecal pH, a decrease in formation of gases, and more gradual formation of SCFA  
10 (including formation in the distal part of the colon), i.e. overall an optimal pattern of fermentation products (more L-lactate, less butyrate etc.). All these changes make the colonic environment more resemble that of breast fed babies. In one embodiment of the invention the use of mixtures of GOS and polyfructose in effective amounts for the preparation of compositions which lead to a colonic environment essentially similar to  
15 that of breast fed infants is, therefore, provided herein.

The beneficial effects found were significantly more pronounced upon co-administration than when either GOS or polyfructose (e.g. TOS or inulin) alone was administered, indicating that GOS and polyfructose act synergistically. In the prior art  
20 no studies were carried out wherein the individual effects of TOS and inulin administration were directly compared to the co-administration of these two types of compounds and clearly such independent individual studies cannot be compared to one another and are unsuitable to identify synergistic effects.

25 This novel synergistic interaction of GOS and polyfructose (e.g. TOS and inulin), and the *in vivo* effects of co-administration thereof, leads to new uses of compositions comprising both GOS and polyfructose in suitable (synergistic) amounts, such as treatment or prevention of colic and/or abdominal cramps, abdominal bloating, flatulence, abdominal pain, constipation, IBS, IBD, allergy and/or increased muco-  
30 protection of the intestine. In addition, a composition comprising lactate (D-lactate and/or L-lactate, preferably L-lactate) may be used to treat or prevent one or more of these symptoms or disorders.

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Thus, unexpectedly, fermentation of the specific mixtures of prebiotics (GOS and polyfructose) by the intestinal flora results in an enhanced and kinetically advantageous formation of SCFA and lactate and an improved pattern of metabolic end products. As a consequence many beneficial effects result, such as effects on mucus production, anti-inflammatory effects, effects on pain perception and effects on abdominal cramps/colic (both via relaxation of the colon and decrease of spontaneous contractions). The nutrition compositions comprising these prebiotic mixtures can also be used for adults having intestinal problems such as IBD or IBS or a decreased intestinal barrier integrity due to malnutrition.

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#### Definitions

"Polysaccharides" refers to carbohydrate chains of monosaccharide units with a chain length of at least 10 units. In contrast, "oligosaccharides" have a chain length of less than 10 units.

15 "Degree of polymerisation" or "DP" refers to the total number of saccharide units in an oligo- or polysaccharide chain. The "average DP" refers to the average DP of oligosaccharides or polysaccharide chains in a composition, without taking possible mono- or disaccharides into account (which are preferably removed if present). The average DP of a composition is used to distinguish between compositions. In addition

20 the % saccharide units, such as the % glucose and % fructose units, in a composition are distinguishing.

"Polyfructose" or "polyfructan" or "fructopolysaccharide" refers to a polysaccharide carbohydrate comprising a chain of  $\beta$  linked fructose units with a degree of polymerisation of 10 or more and comprises, for example, inulin (e.g. inulin HP), levan

25 and/or a "mixed type of polyfructan" (see below).

"Inulin" or "non-hydrolysed inulin" or "inulin HP" is used herein to refer to glucose-terminated fructose chains with the majority of chains (at least 90%, preferably at least 95%) having a degree of polymerisation (DP) of 10 or more. Inulin can thus be described as  $GF_n$ , wherein G represents a glucosyl unit, F represents a fructosyl unit

30 and n is the number of fructosyl units linked to each other, n being 9 or more. The G/F ratio is about 0.1 to 0. A small part of the inulin molecules, however, may have no terminal glucose unit. The average DP is preferably at least 15, more preferably 20 or more, such as 20, 21, 22, 23, 25, 30, 40, 60, 70, 100, 150 or more herein. In inulin the

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fructose units are linked with a  $\beta(2\rightarrow1)$  linkage. A suitable inulin is for example the commercially available as Raftiline®HP (Orafti) with an average DP > 23.

5 “Hydrolised inulin” or “oligofructose” refers to mixtures of glucose- and fructose-terminated fructose chains, with a DP below 10. Thus, hydrolysed inulin can be described as a mixture of  $GF_n$  chains and  $F_n$  chains (wherein G is a glucosyl unit, F is fructosyl unit and  $n = 1-8$ ). Hydrolysed inulin is an (enzymatic or acidic) hydrolysis product or partial hydrolysis product of inulin, resulting from cleavage of  $\beta(1-2)$  fructosyl-fructose linkages. The term hydrolysed inulin also encompasses synthetically  
10 made or recombinantly made inulin which have the same structural makeup.

“Levan” or “levulan” or “levulin” or “levulosan” refers to a polysaccharide consisting of polyfructose in which the fructose units are linked with  $\beta(2\rightarrow6)$  linkages. A starting glucose moiety can be present, but this is not necessary. The degree of polymerisation  
15 is above 10.

In “mixed type polyfructans” the fructose units are linked with  $\beta(2\rightarrow1)$  and  $\beta(2\rightarrow6)$  linkages. Mixed type polyfructans are branched and have a DP > 10.

20 “GOS” or “galactooligosaccharides”, or “trans-galactooligosaccharides” or “TOS” refers to oligosaccharides composed of galactose units, with a DP of 10 or less and an average DP of 2, 3, 4, 5 or 6. A glucose unit may be present at the reducing end of the chain. Preferably the GOS contains at least 2/3 galactose units. Most preferred are trans-galactooligosaccharides (TOS) with  $\beta(1-4)$  glycosidic bonds. Such a GOS is for  
25 example that found in Vivinal®GOS (commercially available from Borculo Domo Ingredients, Zwolle, Netherlands), comprising trans-galactooligosaccharides with  $\beta(1-4)$  glycosidic bonds and  $\beta(1-6)$  glycosidic bonds.

30 “SCFA” or “short chain fatty acids” refers to fatty acids, with a carbon chain lengths of up to C6, produced as an end-product of bacterial intestinal fermentation, such as acetate (C2), propionate (C3), butyrate and isobutyrate (C4), valerate and isovalerate (C5) and others. The expression “iC4-5” refers to the sum of isobutyrate, valerate and isovalerate.



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A "synergistically effective amount" refers to an amount of GOS and polyfructose (for example TOS and inulin) which, when co-administered, confers one (or more) specific physiological effects (as described elsewhere herein), whereby the total effect of co-administration is significantly larger than the sum of the effect of individual administration of GOS or polyfructose. For example, if the effect of administration of GOS alone is X and the effect of administration of polyfructose alone is Y, then the effect of co-administration of GOS and polyfructose is larger than X+Y and for example the effect of co-administration of  $\frac{1}{2}$  the concentration of GOS plus  $\frac{1}{2}$  the concentration of polyfructose has an effect larger than  $\frac{1}{2} X + \frac{1}{2} Y$ , etc.

"Co-administration" of two or more substances refers to the administration of these substances to one individual, either in one composition or in separate compositions (kit of parts; as a combined composition) which are administered at the same time (simultaneously) or within a short time-span (separate or sequential use, e.g. within minutes or hours).

"Enteral" refers herein to the delivery directly into the gastrointestinal tract of a subject (e.g. orally or via a tube, catheter or stoma).

The term "comprising" is to be interpreted as specifying the presence of the stated parts, steps or components, but does not exclude the presence of one or more additional parts, steps or components.

"Infant" refers herein to humans aged 0-36 months, preferably 0-18 months, or more preferably 0-12 months.

"Breast fed infants" refers to infants exclusively fed with human breast milk. "Non- or partially breast fed infants" are infants not exclusively fed on human breast milk. This includes infants fed with at least one bottle (about 80ml) of formula milk per day.

"Percentage" or "average" generally refers to percentages of averages by weight, unless otherwise specified or unless it is clear that another basis is meant.

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In one embodiment the present invention provides a number of novel uses of compositions comprising both GOS and polyfructose in suitable (synergistically effective) amounts.

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Uses and methods according to the invention

In the various uses described herein, a subject is preferably a human subject, more preferably an infant, such as a new born infant up to a 12 months old infant. Especially bottle fed or partially bottle fed infants are referred to. Alternatively, a subject may also  
10 be a child, teenager or adult. In particular intestinal problems, such as but not limited to Inflammatory Bowel Disease (IBD), Irritable Bowel Syndrome (IBS), flatulence, abdominal cramps, colic, abdominal bloating, abdominal pain, etc., which may also result in fatigue, depression or moodiness, may be prevented and/or treated using compositions according to the invention. Also, mucosal production may be enhanced  
15 and allergies resulting from a suboptimal functioning of the intestine may be prevented or treated. Also the intestinal barrier function may be improved in patients with an impaired barrier function as a result from malnutrition, surgery, chemotherapy etc.

Compositions comprising both GOS and polyfructose, such as compositions  
20 comprising TOS and inulin, may in one embodiment be used to induce the production of SCFA in the large intestine of a subject and to significantly increase the (intestinal and/or faecal) total amount of SCFA produced following administration and/or to significantly modify the relative amounts of (intestinal and/or faecal) SCFA produced, in particular to increase the (intestinal and/or faecal) relative amount of acetate out of  
25 total SCFA, and/or to significantly increase the amount (absolute and relative) of intestinal lactate produced, and/or to significantly reduce or prevent intestinal gas formation, and/or to extend the SCFA production to the distal part of the colon, and/or to significantly reduce the pH in the large intestine and faecal pH. Also, the sum of intestinal and/or faecal butyrate, isobutyrate, valerate and/or isovalerate relative to the  
30 total SCFA may be decreased. These effects are achieved following administration, without having a significant effect on the total and/or relative number of intestinal bifidobacteria. Compositions for the treatment or prophylaxis of any diseases or

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disorders or discomforts associated with or caused by one or more of these intestinal effects are provided herein.

5 The present invention provides the use of galacto-oligosaccharides and polyfructose for the manufacture of a composition for the treatment or prevention of abdominal bloating, gas formation, abdominal pain and/or flatulence. In a further embodiment the present invention provides the use of galactooligosaccharides and polyfructose for the manufacture of a composition for the treatment or prevention of allergy, eczema or atopic diseases. In a further embodiment the present invention provides the use of  
10 galacto-oligosaccharides and polyfructose for the manufacture of a composition for the treatment and/or prevention of colics and/or for the relaxation of contractions of the colon, preferably the tonic and/or phasic contraction. In another embodiment the present invention provides the use galacto-oligosaccharides and polyfructose for the manufacture of a composition for the treatment or prevention of irritable bowel  
15 syndrome or inflammatory bowel disease. In an even further embodiment the present invention provides the use of galacto-oligosaccharides and polyfructose for the manufacture of a composition for the increase of intestinal barrier functioning and/or mucus production in the large intestine.

20 Unless specified otherwise, a "significant increase" (of for example SCFA, acetate or lactate) refers herein to an increase of at least 5%, preferably at least 10%, more preferably at least 20%, or more, compared to the amount produced when either GOS or polyfructose (e.g. inulin) alone are administered. Similarly, unless specified otherwise a "significant reduction" (of for example gas formation) refers to a reduction  
25 of at least 5%, preferably at least 10%, more preferably at least 20%, 25%, 50% or more (e.g. 70%, 80%, 90%, 100%) compared to the amount produced when polyfructose (e.g. inulin) alone is administered.

30 An increase in the total amount of SCFA produced as fermentation product of GOS and polyfructose compositions can also be measured as a significant decrease in pH of faecal samples of subjects, reflecting an *in vivo* decrease in pH in the large intestine. In infants it was found that faecal pH decreased to about pH 5.8 or less, such as pH 5.6, 5.5 or 5.4 or 5.2 following co-administration of TOS and polyfructose (e.g. inulin),

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while the pH of standard formula fed infants was around or above pH 6, such as about pH 5.9, 6.8 or even 7.0. A significant decrease in pH refers therefore to a decrease in pH by at least 15% or 0.9 unit compared to infants fed a standard formula.

5 Another beneficial effect of co-administration of GOS and polyfructose in suitable amounts is that the composition of SCFA is significantly different than when either GOS or polyfructose are administered. Especially the relative amount of butyrate is significantly reduced, while the relative amount of acetate is significantly increased. The compositions may, thus, be used to alter not only total SCFA quantities, but also  
10 relative SCFA proportions. In infants co-administered with TOS and polyfructose (e.g. inulin), the relative SCFA levels resembled more the levels found in breast fed infants, with acetate : propionate : butyrate ratios generally about 80-85% : 10-15% : 1-5%, while in infants fed on standard milk formula ratios were generally around 69-74% : 16-19% : 5-6% ( as shown in the examples). Thus, especially relative amounts of  
15 acetate are increased and relative amounts of propionate and butyrate are decreased by the compositions according to the invention. The co-administration of GOS and polyfructose results in acetate : propionate : butyrate proportions resembling that of breast-fed infants much more closely than that of formula fed infants (which produce high levels of butyrate and propionate and relatively low levels of acetate). The effect  
20 on relative SCFA proportions can be measured by methods known in the art, such as gas chromatography of faecal samples at various time points after administration, either using in vivo studies or an in vitro fermentation systems as e.g. described in the Examples. A "significantly modified SCFA composition" or a "significantly increased amount of acetate" refers to the amount of acetate (% of total SCFA) being at least  
25 about 4%, 5%, 10%, 15%, or more, higher than when no GOS or polyfructose are administered or when GOS or polyfructose are administered individually. Preferably the relative amounts of propionate and/or butyrate are lower than when no GOS or polyfructose are administered or when GOS or polyfructose are administered individually. In one embodiment, the composition according to the invention is suitable  
30 for increasing intestinal and/or faecal acetate to above about 85%, such as 86%, 87%, 88%, 90% or more, of the total SCFA.

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Yet a further beneficial effect observed when co-administering GOS and polyfructose is a significant decrease in branched SCFA, compared to the proportion of branched SCFA found when only GOS or only polyfructose are administered. A "significant decrease in branched SCFA", as used herein, refers to a decrease at least 70% compared to the concentration found in infants not fed prebiotics or resulting in a faecal proportion of less than 1.5% of total SCFA. The proportion of branched SCFA relative to the total SCFA can be measured by dividing the sum of branched SCFAs, i.e. isobutyrate, plus isovalerate plus valerate, by the sum of total SCFA, i.e. acetate, plus propionate, plus butyrate, plus isobutyrate, plus isovalerate, plus valerate, *etc.*.

Reducing the proportion of branched SCFA is beneficial to the health of the subject, as branches SCFA are damaging. This indicates less protein degradation, which is unwanted because protein fermentation results in an increase of pH and in the formation of damaging agents such as H<sub>2</sub>S.

A significant decrease of butyrate relates to a decrease by at least 50% compared to the concentration found in infants not fed prebiotics or resulting in a faecal proportion of less than 4% of total SCFA. In one embodiment the composition according to the invention is suitable for decreasing the sum of intestinal (and/or faecal) butyrate, isobutyrate, valerate and isovalerate is below 7% of total SCFA, such as 6.5%, 6%, 5%, 4% or less of total SCFA.

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In another embodiment, compositions comprising GOS and polyfructose are suitable to increase the length of fermentation within the colon. In particular, bacterial fermentation will still be active in the most distal parts of the colon following co-administration, as indicated by SCFA production in the distal part of the colon. No or only very little SCFA is produced in the distal part of the colon following administration of compositions comprising only GOS or only polyfructose. In addition, fast fermentation at the beginning of the colon is seen following co-administration, which is especially important for anti-pathogenic effects and is also observed in breast fed infants. Overall, this indicates the compositions are suitable for establishing and/or maintaining a relatively even fermentation pattern throughout the colon of a subject and to extend fermentation and SCFA production to the distal end of the colon. SCFA, and most likely also other fermentation products, especially lactate, are therefore produced throughout the colon, in the beginning, middle and end of the colon.

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In a further embodiment, co-administration of GOS and polyfructose in synergistic amounts may be used for significantly increasing lactate production, as can be determined again by analysing faecal samples of test subjects and control subjects, which received compositions comprising either GOS alone or polyfructose alone or equivalent base compositions without prebiotics. A "significant increase in lactate" refers to at least 5%, 10%, 20% or even 50% or more lactate being produced in subjects being co-administered compositions comprising GOS and polyfructose, compared to subjects not administered GOS or polyfructose. For example, infants fed on standard milk formula supplemented with 6 g/l of TOS/inulin mixtures (90% : 10%) produced at 16 weeks about 16% lactate (as % of total acids), while standard formula fed infants produced about 0.6% and breast fed infants produced about 35% lactate. Although not matching the amount of lactate produced by breast fed infants, the supplementation of the standard milk formula with TOS and inulin mixtures led to a significant increase in lactate production. As shown in the Examples, lactate was found to have unexpected beneficial effects. Similarly to acetate it increased the prostaglandin E1 and prostaglandin E2 production and resulted in enhanced epithelial mucin expression. The intestinal mucosa plays an important role in human health, as it serves as a barrier to infectious pathogens, allergens and carcinogens. Compositions which positively influence the development and/or integrity of the mucosa may therefore be used to aid the build up of a healthy mucosa in new born infants and to aid the establishment of a healthy mucosa in bottle-fed or partially bottle-fed infants and in subjects suffering from symptoms or diseases resulting from or being associated with a not properly functioning intestinal mucosa. For example, intestinal problems, such as Inflammatory Bowel Disease (IBD, for example colitis or Crohn's disease) or Irritable Bowel Syndrome (IBS) may be treated or prevented, or pathogen attachment and/or entry through the mucosa may be reduced or other immunological problems, such as the development of asthma, may be prevented or reduced. Also the loss of intestinal barrier integrity as a result from malnutrition, surgery, chemotherapy etc. can be prevented or reduced.

Furthermore, spontaneous contractions and abdominal tension was significantly reduced in a dose dependent manner following D- or L-lactate administration. The

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compositions according to the invention may thus be used to increase intestinal lactate levels and thereby treat or prevent symptoms such as abdominal pain, colic, abdominal contractions, abdominal tension and the like.

5 As lactate itself was found to have previously unknown effects, it is also an object of the invention to provide compositions comprising lactate in suitable amounts, which may be used to treat or prevent the above described symptoms and disorders. In one embodiment compositions comprises only lactate in suitable amounts, while in another embodiment the GOS an polyfructose compositions described herein further comprises  
10 lactate to further increase intestinal lactate levels. One such composition according to the invention comprises TOS and inulin in synergistically effective amounts and further comprises lactate in suitable amounts. In one embodiment compositions are provided which are suitable for increasing intestinal and/or faecal lactate to above about 10 mmol/kg faeces.

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It is understood that when referring to the analysis of faecal samples this is an indirect measure of the actual *in vivo* effect. Likewise, *in vitro* assays may be carried out, whereby bacterial populations are grown *in vitro* and suitable amounts of compositions are added to the cultures. In particular, the *in vitro* fermentation system described in the

20 Examples may be used.

Co-administration of compositions comprising GOS and polyfructose can therefore be used to achieve one or more of the following physiological effects:

- a significant increase in total SCFA
- 25 - a significant decrease in pH
- a significantly modified SCFA composition
- a significant decrease in branched SCFA
- a significant decrease in gas production per mol SCFA produced
- a longer and more even fermentation, including fermentation even in the most distal  
30 parts of the colon, and/or
- a high initial formation of SCFA and /or lactic acid formation in the most proximal part of the colon
- promoting the production of SCFA at the beginning, middle and end of the colon

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- a significant increase in lactic acid (absolute and/or relative) and/or
- an increase in the relative amount of L-lactate as a ratio of total lactic acid formed.

In a preferred embodiment at least 2, 3, 4, 5, 6, 7, 8, 9 or all these effects are significantly modified when using compositions comprising both GOS and polyfructose, compared to when administering compositions comprising only GOS or polyfructose, or neither GOS or polyfructose. In particular, at least 2, 3, 4, 5, 6, 7, 8, 9 or all these effects are significantly larger following co-administration of a (synergistically effective amount) of GOS and polyfructose than the sum of the effect(s) of administration of GOS and polyfructose individually.

The synergistic compositions according to the invention are, therefore, particularly suited to build up and/or maintain a healthy microflora within an infant's large intestine following administration of a synergistically effective amount. In a preferred embodiment an infant is fed solely on a base composition, such as milk formula, supplemented with a synergistically effective amount of GOS and polyfructose within the first weeks after birth. A preferred composition is, therefore, a dietary composition for infants. In another embodiment an infant is only partly fed on a composition according to the invention. In one embodiment the composition is used to treat or prevent symptoms selected from one or more of: gas formation, flatulence, colitis, abdominal bloating, abdominal cramps, abdominal pain, IBS, IBD, allergy and/or as a mucoprotectant. GOS and polyfructose may, thus, be used for the preparation of a composition or a combined composition for simultaneous, separate or sequential use in treatment of flatulence, excessive gas formation, colitis, abdominal bloating, abdominal cramps, abdominal pain, IBS, IBD, allergy, decreased intestinal barrier functioning and/or as a mucoprotectant. The subject may in this case be any subject, ranging from infant, child, teenager to adult. Clearly, the base compositions to which GOS and polyfructose are added in synergistically effective amounts, will vary depending on the age of the subject, the mode of administration and on the main symptoms to be treated or prevented. For infants for example the base composition is preferably a liquid or powder form infant or follow-on formula, while for adults a nutrient supplement composition (liquid, semi-liquid or solid) or tube feeding may be more suitable.



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In a preferred embodiment GOS and polyfructose are co-administered in a synergistically effective amount suitable to significantly increase the total amount of intestinal SCFA formed and/or to significantly improve intestinal SCFA composition (especially to significantly increase the percentage of acetate relative to the total amount of SCFA) and/or to significantly increase lactate production and/or to and to lower intestinal pH and/or to significantly reduce intestinal gas formation.

Use of compositions comprising a synergistically effective amount of GOS and polyfructose for the treatment and/or prevention of colic and/or abdominal cramps, abdominal bloating and/or flatulence, abdominal pain, IBS, IBD and/or allergy is provided. Use of such compositions for increasing mucoprotection and strengthening the intestinal barrier is also provided. It is understood that not necessarily one composition is referred to, but that GOS and polyfructose may be present in separate compositions, which provides a synergistically effective amount when co-administered.

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Through these changes in SCFA levels, profile and intestinal pH, the compositions are able to cause one or more of the following downstream beneficial effects:

- a) The intestinal permeability at the site of SCFA production is decreased. This is important for preventing disease and maintaining health, especially to prevent allergies from developing. The finding that co-administration of GOS and polyfructose causes more even fermentation and extends fermentation to the distal part of the colon is important in this respect, as the permeability of the intestine, including the distal parts of the colon, can be evenly reduced and the intestine can thereby be evenly maintained in a healthy state.
- b) The motility or peristaltic movement of the intestine is enhanced, which reduces or prevents constipation, a common problem observed in formula fed infants.
- c) Decreasing the amount of spontaneous contractions and the colonic muscle tension resulting in less cramps and less abdominal pain.
- d) Calcium-ion absorption is increased, which is important for bone mineralisation and bone development of the subject, especially if the subject is an infant
- e) Secondary symptoms of reduced health such as fatigue, depression and mood fluctuation may be reduced,

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- f) Mucus production of the intestinal mucosa is significantly enhanced, which provides protection against pathogen attachment and colonization. In particular, it was found that lactate levels correlated positively with mucus production. The compositions according to the invention may thus be used to stimulate mucus production.
- g) Bile metabolism may be modified to levels and/or patterns as observed in breast fed infants.
- h) Growth of intestinal pathogenic microorganisms is inhibited along the entire colon.
- 10 A composition comprising a synergistically effective amount of GOS and polyfructose may therefore be used to either maintain or establish (e.g. in a newborn, premature or mature born baby or in a non- or partially breast fed baby, or other subjects, such as adults, one or more of the above physiological effects and maintain or improve the health of the subject by ensuring or establishing a healthy large intestinal environment and optimal activity of the large intestine.

In particular, incidence, duration and/or severity of diseases, disorders or symptoms such as colic and/or abdominal cramps, abdominal bloating and/or flatulence, abdominal pain, IBS, IBD and/or allergy, intestinal pathogen infection (e.g. bacteria, viruses, fungi), diarrhoea, eczema, allergy induced asthma and other atopic diseases, and/or constipation can be reduced, abolished or prevented by administration of a composition according to the invention. The compositions according to the invention may also be used to relax contractions of the colon, preferably the phasic and/or tonic contractions.

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#### Compositions according to the invention

Compositions suitable for the uses described above comprise both polyfructose and GOS in synergistically effective amounts. The compositions comprise GOS/polyfructose in ratios ranging from 3/97 to 97/3, preferably 5/95 to 95/5, more preferably 90/10 to 45/55. All individual ratios between these end-points are encompassed herein, such as 10/90, 20/80, 30/70, 40/60, 50/50, 55/45, 60/40, 70/30, 80/20, 85/15, 90/10, etc.

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The dosages required and the GOS / polyfructose ratio for achieving the (optimal) synergistic effect may vary, depending on the type of composition and the method and frequency of administration. A few examples are however given below. A skilled person may easily determine what dosages of each component is required to achieve the best physiological effect and what GOS / polyfructose ratio is the most suitable. For example, if the main aim is to enhance the total level of SCFA following oral administration of a composition comprising GOS and polyfructose, a skilled person will make various compositions (comprising GOS plus polyfructose, GOS alone, polyfructose alone) and compare their efficiency in inducing high SCFA levels using either *in vivo* or *in vitro* tests as known in the art. It is understood that when referring to "daily dose" or 'dosage per day', this does not imply that the dosage must be administered to the subject at one time.

A synergistically effective infant formula may, for example, consist of a base composition (e.g. standard infant formula) comprising about 4 g/l, 5 g/l or more of a mixture of GOS and polyfructose, whereby the ratio's of GOS to polyfructose may vary as described elsewhere herein (e.g. 90% GOS : 10% polyfructose).

Polyfructose may, for example, be levan and/or inulin. Polyfructose, such as levan or inulin, for use in the compositions may be either extracted from natural sources (plants or bacteria) or may be made by *de novo* synthesis or by recombinant DNA technologies as known in the art. Extraction, size separation and purification methods of inulin have been described, for example in De Leenheer (1996), US6569488 and US5968365. Recombinant production methods have been for example described in US6559356. Synthesis generally involves the use of sucrose molecules and enzymes with fructosyl transferase activity. Inulin suitable for use in the compositions is also already commercially available, e.g. Raftiline®HP, Orafiti.

Plant inulins generally have a much lower degree of polymerisation than bacterial inulins (up to 150, compared to up to 100,000 in bacteria). Plant sources include dicotyledenous species, such as *Compositae*. Examples of species which produce relatively large amounts of inulin, mainly in their roots, bulbs or tubers, are chicory, asparagus, dahlia, Jerusalem artichoke, garlic, and others (see Kaur and Gupta, J.

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Biosci. 2002, 27, 703-714). As the inulin should preferably not comprise oligofructose, hydrolysis should be avoided or oligofructose and/or mono- or disaccharides present should be removed prior to use.

- 5 Especially preferred is polyfructose with a PD of at least 10, 15, 20, 50, 70, 100, 120, 130, 150, 200, 300, 500 or more. Polyfructoses, such as levans, may be hydrolysed to avoid viscosity problems associated with too long chains, as long as a PD of 10 or more is retained.
- 10 Levans may also be obtained from natural sources such as plants (e.g. monocotyledons) yeast, fungi, bacteria, or made chemically or using recombinant DNA technology.

- GOS may be obtained from natural sources, such as plants (e.g. chicory, Soya) or bacteria, or may be made synthetically or by recombinant DNA technology as known
- 15 in the art. GOS may be  $\beta$ -galacto-oligosaccharide or  $\alpha$ -galacto-oligosaccharide or a mixture of both. In particular, galactose residues are linked by  $\beta(1-4)$  and  $\beta(1-6)$  glycosidic bonds (trans GOS or TOS). GOS suitable for use in the compositions is also already commercially available, e.g. Vivinal®GOS, Borculo Domo Ingredients, Zwolle, The Netherlands. GOS may also be derived from lactose, by treatment of
- 20 lactose enzymatically with  $\beta$ -galactosidase or by hydrolysis from polyglucan. In a preferred embodiment TOS is used. In one embodiment the preferred polyfructose is inulin HP, so that the combination of TOS and inulin HP are also a preferred embodiment herein.

- 25 Other compositions provided are compositions comprising lactate in suitable amounts, in particular for the uses described above. Suitable amounts of lactate may vary, and may for example range from 1 to 30 grams per day.

- 30 Compositions according to the invention may be either food compositions, food supplement compositions or pharmaceutical compositions. Apart from polyfructose and GOS (and/or lactate) they may comprise additional ingredients. For food compositions or food supplement compositions these should be food grade and physiologically acceptable. "Food" refers to liquid, solid or semi-solid dietetic compositions, especially

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total food compositions (food-replacement), which do not require additional nutrient intake or food supplement compositions. Food supplement compositions do not completely replace nutrient intake by other means. Food and food supplement compositions are in a preferred embodiment baby food or food supplements, food or food supplements for prematurely born babies, infant food, toddler food, etc., which are preferably administered enterally, preferably orally several times daily. The food or food supplement compositions are particularly suited for non- or partially breast fed infants. Also, the composition may be beneficially administered to infants in their adaptation period to solid food or infants changing from breast to bottle feeding. The composition may also be part of a human milk fortifier supplement.

In one embodiment the composition is an infant or follow-on formula as e.g. known in the art, especially as described in the European Commission directive 91/321/EEC and the amendments thereof, but may be modified to comprise an effective amount of polyfructose and GOS. The infant or follow-on formula may be based on milk (cows milk, goats milk, etc.), infant milk formula (IMF) or soy for lactose intolerant infants or may contain amino acids as a nitrogen source for infants having problems regarding allergy or absorption. Commercially available infant or follow-on formulae comprise, thus, a milk protein or soy protein base, fat, vitamins, digestible carbohydrates and minerals in recommended daily amounts and may be powders, liquid concentrates or ready-to-feed compositions.

Administration of the modified infant or follow-on formula results in a large intestinal environment which resembles that of breast-fed infants, as can be determined by analyses of faecal pH, bacterial composition, SCFA production and profiles, gas production, etc. When the composition is a drink, preferably the volume (comprising the daily effective dose) consumed or administered on a daily basis is in the range of about 100 to 1500ml, more preferably about 450 to 1000 ml per day. When the formula is a solid preferably the amount (comprising the daily effective dose) consumed or administered on a daily basis is in the range of about 15 to 220 g/day, preferably about 70 to 150 g/day of formula powder.

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A daily effective dose of GOS and polyfructose ranges from about 1 to 30 g/day, preferably from about 2 to 10 g/day for infants and preferably for adults from about 5 to 20 g/day.

- 5 Food or food supplement compositions according to the invention may additionally comprise other active ingredients, such as vitamins (A, B1, B2, B3, B5, B12, C, D, E, K, etc.), probiotics (e.g. bifidobacteria, lactobacilli, etc.), other prebiotics, fibres, lactoferrin, immunoglobulins, nucleotides, and the like. Nutrients such as proteins, lipids and other carbohydrates (e.g. digestible carbohydrates, non-digestible
- 10 carbohydrates, soluble or insoluble carbohydrates) may be present in various amounts. Typical in-soluble non-digestible carbohydrates present in infant nutrition are soy polysaccharides, resistant starch, cellulose and hemicellulose. Typical soluble and digestible carbohydrates for use in infant nutrition are for example maltodextrin, lactose, maltose, glucose, fructose, sucrose and other mono- or disaccharides or
- 15 mixtures thereof. The composition may also comprise other inactive ingredients and carriers, such as e.g. glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. The compositions may also comprise water, electrolytes, essential and non-essential amino acids, trace elements, minerals, fibre, sweeteners,
- 20 flavorings, colorants, emulsifiers and stabilisers (such as soy lecithin, citric acid, esters of mono- or di-glycerides), preservatives, binders, fragrances, and the like. Lipids suitable for the compositions, especially for infant food or food supplements, are milk fats, plant lipids, such as canola oil, safflower oil, sunflower oil, olive oil, marine oils, etc. or fractions or mixtures thereof comprising suitable fatty acids
- 25 (polyunsaturated and/or saturated). Proteins suitable for the compositions especially for infant food or food supplements, include casein, whey, condensed skimmed milk, soy, beef, collagen, corn and other plant proteins or hydrolysed proteins, free amino acids, etc. Preferably proteins comprised in infant food or food supplement compositions are extensively hydrolysed
- 30 and/or partially hydrolysed to reduce the risk of allergies. The infant food compositions according to the invention preferably comprise all vitamins and minerals essential in the daily or weekly diet in nutritionally significant amounts, such as minimal recommended daily amounts.

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- The food or food supplement composition may also in one embodiment be made on the basis of (i.e. starting from or comprising) a food base. It may, for example, based on, or comprises, a dairy product, such as a fermented dairy product, including but not limited to milk, yoghurt, a yoghurt-based drink or buttermilk. Such compositions may be prepared in a manner known per se, e.g. by adding an effective amount of polyfructose and GOS or TOS to a suitable food or food base. Other food bases suitable may be plant bases, meat bases and the like.
- 10 The food or food supplement composition according to the invention may be used either as a treatment and/or prophylactically. This is to say that they may be either administered after gastrointestinal problems or diseases have been diagnosed in a subject or, alternatively, prior to the occurrence of symptoms (for example to high risk patients, likely to develop gastrointestinal problems). For example, if symptoms associated with the disease or suboptimal functioning of the large intestine, such as constipation, flatulence, allergies, colics, abdominal pain, abdominal cramps, IBD, IBS are observed, administration of the composition will aid in re-establishing a healthy large intestinal environment or prevent the development of such symptoms.
- 20 In one embodiment the compositions are administered prophylactically, to support the development of a healthy microflora and/or healthy large intestinal environment. A "healthy large intestinal environment" refers to a normal intestinal physiology and activity, especially normal absorption of nutrient, water, resistance of the mucosa to pathogen attachment, colonisation and infection, etc. as can be determined by faecal analysis and gas production. Especially, an optimal physiology and activity is referred to. Any suboptimal functioning of the large intestine results in symptoms as described and can be determined by faecal analysis and/or gas production. Intestinal diseases or disorders resulting from a suboptimal functioning are included herein.
- 30 Pharmaceutical compositions for the treatment or prophylaxis of intestinal disorders or symptoms of suboptimal intestinal functioning, such as IBD, colitis, IBS and/or increased barrier integrity may comprise additional biologically active ingredients, such as drugs, biologically active proteins or peptides, probiotics, and others. The

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embodiments described for food or food supplement compositions apply also to pharmaceutical compositions.

5 The compositions according to the invention may be in any dosage form, such as liquid, solid, semi-solid, tablets, drinks, powders, etc., depending on the method of administration. Administration to a subject is preferably oral, although for some uses rectal or tube feeding (with a tube directly entering the stomach, duodenum, or small intestine or large intestine) may be suitable.

10 It is a further embodiment of the invention to provide a method for the manufacture of a composition according to the invention by adding a synergistically effective amount of GOS (or TOS) and polyfructose to a suitable composition base, as described above.

15 The following non-limiting Examples describe the synergistic effect of GOS and polyfructose. Unless stated otherwise, the practice of the invention will employ standard conventional methods of molecular biology, pharmacology, immunology, virology, microbiology or biochemistry. Such techniques are described in Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, NY, in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology*, *Current Protocols*, USA and Remington's  
20 Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985), *Microbiology: A Laboratory Manual* (6th Edition) by James Cappuccino, *Laboratory Methods in Food Microbiology* (3<sup>rd</sup> edition) by W. Harrigan (Author) Academic Press, all incorporated herein by reference.

25

#### Figure Legends

##### Figure 1

Formation of acetate, propionate and butyrate after 48 h *in vitro* fermentation of TOS, Inulin, a mixture of TOS/Inulin 9/1 and a mixture of oligofructose and inulin 1/1 by  
30 fresh faeces obtained from babies.

##### Figure 2

Relative amounts of acetate, propionate and butyrate formed after 48 h *in vitro* fermentation of TOS, Inulin, a mixture of TOS/Inulin 9/1 and a mixture of



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oligofructose and inulin 1/1 by fresh faeces obtained from babies. The sum of acetate plus propionate plus butyrate formed was set at 100 % for each of the fibre tested.

### Figure 3

5 Formation of gas after 48 h *in vitro* fermentation of TOS, Inulin, a mixture of TOS/Inulin 9/1 and a mixture of oligofructose and inulin 1/1 by fresh faeces obtained from babies. The amount of gas formed (ml) was related to the amount of total SCFA formed (in mmol per g fibre)

### Figure 4

10 Effects of mixtures of SCFA (acetate/propionate/butyrate in a ratio of 90/5/5 on a molar basis) and of L-lactate.

Figure 4A: Effect of 0, 0.1, 0.5, 1 and 4 mM of the SCFA mixture or L-lactate on muc-2 expression in a CCD18/T84 co-culture (n=6). Error bars show the SEM. The increase at 1 and 4 mM SCFA mixture and 0.5, 1 and 4 mM L-lactate is statistically significant.

15 Figure 4B: Effect of 0, 50, 100, 250 and 500  $\mu$ M SCFA mixture or L-lactate on the spontaneous PGE1 and PGE2 response in CCD18 cells (n=7). Error bars show the SEM. The increase of PGE1 at 100, 250 and 500  $\mu$ M SCFA mixture and 100  $\mu$ M L-lactate is statistically significant. The increase of PGE2 at 100, 250 and 500  $\mu$ M SCFA mixture and 250  $\mu$ M L-lactate is statistically significant (P<0.05)

### Figure 5

20 Effects of sodium acetate and sodium L-lactate on the spontaneous contraction in the distal and proximal part of the colon. The blanc (set to a tension of 1 g) is 0%. The tension after addition of 40 mM KCl was set to 100%.

## Examples

### 25 Example 1

*In vitro* fermentation studies show synergistic effects on fermentation patterns

#### 1.1 Materials and methods

##### Microorganisms

30 Microorganisms were obtained from fresh faeces from bottle fed babies. Fresh faecal material from babies ranging 1 to 4 month of age was pooled and put into preservative medium within 2 h.

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Compositions/Substrate

As substrate either prebiotics (TOS; TOS (from VivinalGOS, Borculo Domo Ingredients, The Netherlands) and inulin (rafilinHP from Orafiti, Belgium)mixture in a 9/1 (w/w) ratio; inulin; oligofructose and inulin mixture in a 1/1 (w/w) ratio, or none  
5 (blanc) was used.

Media

McBain & MacFarlane medium: Buffered peptone water 3.0 g/l, yeast extract 2.5 g/l, mucin (brush borders) 0.8 g/l, tryptone 3.0 g/l, L-Cysteine-HCl 0.4 g/l, bile salts 0.05 g/l,  $K_2HPO_4 \cdot 3H_2O$  2.6 g/l,  $NaHCO_3$  0.2 g/l, NaCl 4.5 g/l,  $MgSO_4 \cdot 7H_2O$  0.5 g/l,  $CaCl_2$   
10 0.228 g/l,  $FeSO_4 \cdot 7H_2O$  0.005 g/l. 500 ml Scott bottles were filled with the medium and sterilised for 15 minutes at 121°C.

Buffered medium:  $K_2HPO_4 \cdot 3H_2O$  2.6 g/l,  $NaHCO_3$  0.2 g/l, NaCl 4.5 g/l,  $MgSO_4 \cdot 7H_2O$ ,  
0.5 g/l,  $CaCl_2$  0.228 g/l,  $FeSO_4 \cdot 7H_2O$  0.005 g/l. pH was adjusted to  $6.3 \pm 0.1$  with  
15  $K_2HPO_4$  or  $NaHCO_3$ . 500ml Scott bottles were filled with the medium and sterilised for 15 minutes at 121°C.

Preservative medium: Buffered peptone 20.0 g/l, L-Cysteine-HCl 0.5 g/l, Sodium thioglycollate 0.5 g/l, resazurine tablet 1 per litre. pH was adjusted to  $6.7 \pm 0.1$  with 1  
20 M NaOH or HCl. The medium was boiled in microwave. 30 ml serum bottles were filled with 25 ml medium and sterilised for 15 minutes at 121°C.

The fresh faeces were mixed with the preservative medium. Fresh faeces can be preserved in this form for several hours at 4°C.

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Faecal suspension: The preserved solution of faeces was centrifuged at 13,000 rpm for 15 minutes. The supernatant was removed and the faeces was mixed with the McBain & Mac Farlane medium in a weight ratio of 1:5.

30 Fermentation

3.0 ml of the faecal suspension were combined with 85 mg glucose or prebiotic or with no addition (blanc) in a bottle and mix thoroughly. A  $t=0$  sample was withdrawn (0.5 ml). 2.5 ml of the resulting suspension was brought in a dialysis tube in a 60 ml bottle

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filled with 60 ml of the buffered medium. The bottle was closed well and incubated at 37°C. Samples were taken from the dialysis tube (0.2 ml) or from the dialysis buffer (1.0 ml) with a hypodermic syringe after 3, 24, and 48 hours and immediately put it on ice to stop fermentation.

5

#### Determination of the short chain fatty acids and lactate

See Example 2. Values were corrected for blanc.

#### Gas determination

- 10 At t=3, t=24 and t=48 the gas pressure in the head space of the 60 ml bottle was measured by a gas pressure meter (Druckmessumformer, Econtronic, Germany) by stinging a hypodermic 6 ml syringe through the rubber cap of the bottle and withdrawal of gas from the headspace by this syringe until the gas pressure was 0 bar. The volume in the syringe was the volume of gas formed. Values were corrected for blanc.

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#### 1.2 Results

*In vitro* fermentation was carried out using the following samples:

- 1.) 85mg TOS (from VivinalGOS, Borculo Domo Ingredients, The Netherlands)
- 2.) 85 mg inulin (RaftilinHP, from Orafit, Belgium)
- 20 3.) 85 mg TOS/inulin with a ratio of TOS/inulin of 9/1 (w/w) and
- 4.) 85 mg OF (raftiloseP95, from Orafit, Belgium)/inulin (raftilinHP) in a ratio of OF/inulin of 1/1 (w/w).

#### Total amount of SCFA produced

- 25 Results are shown in Figure 1. Figure 1 shows that the mixture of TOS/Inulin resulted in a significantly higher amount of SCFA per g fibre than the single components, but also higher than the mixture of oligofructose (OF) and inulin. Also tested were 85 mg TOS/inuline in a ratio of 1/1 (data not shown), which also provided a synergistic effect.

#### 30 L- and D-lactate production

L-and D-lactate could only be determined at t=3. Table 1 shows the metabolic end products formed at that time point.

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Table 1 – metabolic end-products (mmol/g fibre) formed after 3 hours *in vitro* fermentation

		Acetate	Propionate	Butyrate	L-lactate	D-lactate
5	TOS	0.23	0	0	0.14	0.03
	Inulin	0	0	0	0.00	0.00
	TOS/Inulin	0.40	0	0	0.17	0.04
	OF/Inulin	0.30	0	0	0.04	0.02

- 10 Again a synergistic higher formation of lactate is observed for the mixture TOS/Inulin compared to the single components TOS and Inulin. Compared to the mixture with OF and Inulin the percentage lactate (based on total acids) and ratio L-/D- lactate is higher in the TOS/Inulin mixture.

#### 15 Relative amounts of SCFA

Figure 2 shows the pattern of fermentation products after 48 h. The mixture of TOS/inulin shows a significantly higher percentage of acetate than the single components, which is also higher than for the mixture of oligofructose (OF) and Inulin. Faeces of breast fed babies show a high percentage of acetate, so the mixture of

- 20 TOS/Inulin results in a pattern of fermentation products which most resembles that of breast fed babies.

#### Gas formation

Results are shown in Figure 3. Regarding the formation of gas, TOS and the mixture

- 25 TOS/Inulin form the lowest amount of gas per mmol SCFA formed. Inulin and the mixture of OF/inulin show a much higher amount of gas formation. Per mmol SCFA formed the amount of gas is lowest in the TOS/inulin mixture.

#### Kinetics of SCFA formation

- 30 Table 2 shows the kinetics of SCFA formation. The combination of TOS/inulin still shows a high SCFA formation between 24 and 48 h, indicating that in the distal part of the colon still SCFA is formed and having a beneficial effect on the colon permeability, mucus formation and anti-pathogenic effects etc. Also in the first 3 h the highest

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amount of SCFA is formed, as is the case with human milk oligosaccharides (data not shown). A fast fermentation at the beginning of the colon is of importance because of the antipathogenic effects.

5 Table 2: kinetics of SCFA formation (mmol) SCFA (blanc corrected)

	Time interval (hours)		
	0-3 hrs	3-24 hrs	24-48 hrs
Prebiotics			
TOS	0.23	3.85	0.13
TOS/inulin HP	0.40	4.49	0.24
Inulin HP	0.00	3.05	0.05
OF/Inulin HP	0.11	4.26	0.00

OF = oligofructose

### Example 2

10 Clinical study with TOS/Inulin: A relative increase of acetate and relative decrease of butyrate is not correlated with an increase of Bifidobacteria. TOS and Inulin have a synergistic effect.

### 2.1 Materials and methods

63 pregnant woman who had decided to breast-feed and 57 who chose not to, were recruited during their last trimester of pregnancy. Infants with normal birth weight, no congenital abnormality, congenital disease or gastrointestinal disease were enrolled within 3 days after delivery. The study was approved by the ethical committee of the Medical Center, St. Radboud, Nijmegen, The Netherlands. Written informed consent was obtained from the parents before enrolment in the study.

20 Infants of mothers who had decided not to breast-feed, were randomly and double blindly allocated to one of two formula groups (OSF, SF). The standard formula group (SF; n=19 received a regular, non-supplemented infant formula (Nutrilon I, Nutricia, The Netherlands). The main compositional data of the standard formula at standard dilution of 131 g/l are given in Table 3. The prebiotic formula group (OSF; n=19)

25 received the same standard infant formula supplemented with a mixture of 6 g/l transgalacto-oligosaccharides (TOS; Vivinal GOS, Borculo Domo Ingredients, Zwolle, The Netherlands) and inulin (PF; RaftilineHP, Orafit active food ingredient, Tienen,

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Belgium). The mixture comprised 90% TOS and 10% inulin (polyfructose). The study formulas were fed *ad libitum* during the study period. Mothers who decided to breast feed were stimulated to continue breast feeding during the course of the study and were supported by a lactation consultant when needed. At termination of breast feeding their

5 infants received one of two formulae. Compliance was assessed by counting the number of unused formula tins during each visit and comparing the amount of consumed formula with the recorded food intake.

Table 3: Composition of the standard formula per litre

Energy	kcal	670
Protein	g	14
Casein/whey ratio		40/60
Fat	g	35
Total carbohydrates	g	75
Lactose	g	75
Minerals		
Calcium	mg	540
Phosphorus	mg	270
Magnesium	mg	50
Sodium	mg	190
Potassium	mg	680
Chloride	mg	430
Iron	mg	5
Zinc	mg	5

10

Questionnaires

Demographic, clinical and anthropometrical data of the mother are collected prior to delivery. Information on delivery was obtained from the mothers at day 5 after delivery. Information of the infants' food intake, formula tolerance, stool

15 characteristics, health and anthropometrics was obtained from questionnaires at postnatal day 5, 10, 28 and once every 4 weeks thereafter until the end of the study.

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Faecal samples

Parents were asked to take faecal samples from their infants, at postnatal day 5, 10, 28 and once every 4 weeks thereafter. The samples were taken from the diaper, as soon as possible after defecation, collected in faeces containers (Greiner Labortechnik, the Netherlands) and stored immediately at  $-20^{\circ}\text{C}$  by the parent and transported in a portable freezer to the laboratory by the investigators.

Preparation of faecal samples:

For the determination of SCFA, 1 gram of the samples was thawed in ice water diluted 10x in MilliQ and homogenised for 10 minutes using a stomacher (IUL Instruments, Barcelona, Spain). 350  $\mu\text{l}$  homogenised faeces was mixed with 200  $\mu\text{l}$  5% (v/v) formic acid, 100  $\mu\text{l}$  1.25 g/l 2-ethylbutyric acid (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 350  $\mu\text{l}$  MilliQ. The samples were centrifuged for 5 minutes at 14,000 rpm to remove large particles and the supernatant was stored at  $-20^{\circ}\text{C}$ . For the FISH analysis and lactic acid measurements, the samples were thawed in ice water, diluted 10x (w/v) in phosphate buffered saline, pH 7.4 (PBS) and homogenized for 10 minutes using a stomacher. The homogenised faeces were stored at  $-20^{\circ}\text{C}$ .

Fluorescence *in situ* hybridisation

FISH analysis was performed as described (Langendijk *et al*, 1995, Appl. Environ. Microbiol. 61:3069-3075.) with some slight modifications. Paraformaldehyde fixed samples were applied to gelatin coated glass slides (PTFE coated 8-wells [1 cm<sup>2</sup>/well] object slides, CBN labsuppliers, Drachten, The Netherlands) and air-dried. The dried samples were dehydrated in 96% ethanol for 10 minutes. Hybridisation buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.1% SDS [pH 7.1]) with 10 ng/l Cy3 Labeled *Bifidobacterium* specific probe Bif164mod (5'-CAT CCG GYA TTA CCA CCC), was preheated and added to the dried samples. Bif 164 mod is modified version of probe S-G-Bif-a-0164-a-A-18 ((Langendijk *et al*, 1995, Appl. Environ. Microbiol. 61:3069-3075.). The slides were incubated overnight in a dark moist chamber at  $50^{\circ}\text{C}$ . After hybridisation the slides were washed for 30 minutes in 50 ml preheated washing buffer (20 mM Tris-HCl, 0.9 M NaCl [pH 7.2]) and briefly rinsed in MilliQ. For staining all bacteria, the samples were incubated with 0.25 ng/l 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 minutes at room temperature. After DAPI staining the slides were briefly rinsed in

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MilliQ, dried, mounted with Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) and covered with a coverslip. The slides were automatically analysed using an Olympus AX70 epifluorescence microscope with automated image analysis software (Analysis 3.2, Soft Imaging Systems GmbH, Münster, Germany). The percentage of bifidobacteria per sample was determined by analysing 25 randomly chosen microscopic positions. At each position the percentage of bifidobacteria was determined by counting all cells with a DAPI filter set (SP 100, Chroma Technology Corp., Brattleboro, U.S.A.) and counting all bifidobacteria using a Cy3 filter set (41007, Chroma Technology, Brattleboro, U.S.A.).

10

#### Short chain fatty acids analysis

The short chain fatty acids (SCFA) acetic, propionic, n-butyric, iso-butyric and n-valeric acids were quantitatively determined by a Varian 3800 gas chromatograph (GC) (Varian Inc., Walnut Creek, U.S.A.) equipped with a flame ionisation detector. 0.5 µl of the sample was injected at 80°C in the column (Stabilwax, 15 x 0.53 mm, film thickness 1.00 µm, Restek Co., U.S.A.) using helium as a carrier gas (3.0 psi). After injection of the sample, the oven was heated to 160°C at a speed of 16 °C/min, followed by heating to 220°C at a speed of 20 °C/min and finally maintained at a temperature of 220°C for 1.5 minutes. The temperature of the injector and detector was 200°C. 2-ethylbutyric acid was used as an internal standard.

20

#### Lactate analysis

Homogenised faeces was thawed on ice and centrifuged for 5 minutes at 14,000 rpm. 100 µl supernatant was heated for 10 minutes at 100°C to inactivate all enzymes. Lactate was determined enzymatically, using a L-lactate acid detection kit with D- and L-lactate-dehydrogenase (Boehringer Mannheim, Mannheim, Germany). Lactate was only determined in those faecal samples which were large enough.

25

#### pH analysis

After storage at -20°C, faecal samples were thawed and the pH was directly measured in the faeces at room temperature using a Handylab pH meter (Scott Glas, Mainz, Germany) equipped with an Inlab 423 pH electrode (Mettler-Toledo, Columbus, U.S.A.).

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### Data analysis

Prior to the study, power calculations showed that to detect a difference in percentage of bifidobacteria between the intervention formula group and the standard formula group of 30% with a SD of 25%, 13 infants per group should be included. Because of an expected drop out of 30% in the formula groups, more infants than calculated were included in the study. Statistical package SPSS (version 11/0) was used for statistical analysis of the results. All values were checked for normality by visual inspection of the normal probability plots. Differences in percentage bifidobacteria, pH, relative amounts of SCFA and lactate between the groups were tested for significance using analysis of variance. In case of a significant difference ( $p < 0.05$ ), groups were compared by using the Bonferroni *post hoc* test. Because it is not possible to double blind assign breast and bottle-feeding and to ensure adequate randomisation, no statistical analysis were performed to compare the breast-feeding with any of the formula feeding groups. Data from the breast-fed group are only given when the infant was only fed breast milk at that time point.

### 2.2 Results

In total, 120 (-pro group infants were included. 57 infants started on formula feeding directly after birth and were equally divided among the formula groups. Of the 63 infants that were fed breast milk directly after birth, 24 switched to formula feeding before the age of 16 weeks and 5 infants dropped out. The characteristics of the study subjects are shown in Table 4. In the formula groups, 9 infants dropped out of the study within the first 16 weeks after birth (4 in the SF, 5 in the OSF group. Reasons for drop out included: colics, suspicion of cow's milk allergy, constipation and practical problems.

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Table 4 - Characteristics of study objects

		Standard Formula, SF N=19	Prebiotic formula, OSF N=19	Breast milk, BF N=63
Sex	Male	5	12	33
	Female	14	7	30
Place of birth	At home	7	8	40
	Hospital	12	11	23
Mode of delivery	Vaginal	14	16	59
	Caesarean	5	3	4
Birth weight		3600±501	3318±602	3651±601

Faecal bifidobacteria

- The percentages of bifidobacteria in faeces at the age of 5 days, 10 days, 4, 8, 12, and 16 weeks of the feeding groups are shown in Table 5 and the amounts in Table 5. The OSF group tends to have a higher bifidobacteria % than the SF group from total bacterial count at all ages, but the differences were not statistically different. Unexpectedly, the percentage of *Bifidobacteria* in breast fed babies was also relatively low and were in line with the formula fed groups.
- Preliminary data also show an increase in *Lactobacilli* in the BF and OSF group, but the amounts of *Lactobacilli* in the faecal flora are at least one order of magnitude lower than the *Bifidobacteria*, the overall pattern is changes very little.

pH results

- The pH values measured in the faeces of the formula-fed infants are shown in table 6. Lowest pH was found in infants fed on breast milk. Faecal pH of faeces of infants fed the OSF formula were lower than the SF group ( $p < 0.045$  at all ages except day 5).

SCFA results

- The total amount of SCFA in the faeces is shown in Table 5 below.
- The percentage of the different SCFA from total SCFA are shown in Table 6. There are no statistically significant differences found in total SCFA concentration between the formula groups. Also the amount of SCFA is comparable to those of the other feeding

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groups. However, already after 10 days, differences in the SCFA profiles can be seen between infants fed on OSF or breast milk compared to infants fed on standard formula. Infants fed the formula containing GOS and polyfructose and fed breast milk, have higher percentages of acetate and lower percentages of propionate, butyrate, iC4-6 SCFA when compared to infants fed the standard formula.

#### Lactate results

The concentrations lactate (mmol/kg faeces) of all groups are shown in Table 5. Already from 5 days of age, the OS formula (not sign.) and the groups of breast milk have higher amounts of lactate compared to the standard formula group. The relative amount of lactate (as a percentage of the sum of SCFA and lactate) is highest in breast fed babies and lowest in standard formula fed babies. Babies fed a formula containing TOS/inulin have an intermediate relative amount of acetate. The percentage of lactate in OSF fed babies at 16 weeks (relative to total acids) significantly differs from that of SF babies.

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Table 5 - Concentration of lactate and total SCFA (mmol/kg faeces) and *Bifidobacteria* (\*1.10<sup>13</sup>/kg faeces) pH between birth and 16 weeks of age. Mean  $\pm$  SEM. Except for pH, no statistically differences were found.

		Lactate	SCFA	pH	<i>Bifidobacteria</i>
5 days	SF	13.5 $\pm$ 7.7	54.7 $\pm$ 12.6	5.93 $\pm$ 0.15	0.58 $\pm$ 0.49
	OSF	10.7 $\pm$ 4.3	56.5 $\pm$ 7.7	5.49 $\pm$ 0.15	1.20 $\pm$ 2.24
	BF	13.3 $\pm$ 2.8	48.7 $\pm$ 4.4	5.27 $\pm$ 0.07	0.47 $\pm$ 0.39
10 days	SF	4.6 $\pm$ 3.0	62.0 $\pm$ 7.9	6.88 $\pm$ 0.15	0.96 $\pm$ 0.83
	OSF	9.7 $\pm$ 3.6	62.3 $\pm$ 7.2	5.95 $\pm$ 0.20	1.10 $\pm$ 0.99
	BF	15.1 $\pm$ 3.2	54.7 $\pm$ 4.9	5.35 $\pm$ 0.07	0.48 $\pm$ 0.61
4 weeks	SF	2.6 $\pm$ 1.4	68.3 $\pm$ 10.3	6.77 $\pm$ 0.21	0.97 $\pm$ 0.96
	OSF	9.9 $\pm$ 3.4	83.1 $\pm$ 8.8	<u>5.88<math>\pm</math>0.18*</u>	1.20 $\pm$ 0.55
	BF	22.8 $\pm$ 4.4	59.8 $\pm$ 4.8	5.45 $\pm$ 0.12	0.56 $\pm$ 0.64
8 weeks	SF	7.6 $\pm$ 6.8	76.5 $\pm$ 13.2	6.80 $\pm$ 0.20	0.89 $\pm$ 0.56
	OSF	24.4 $\pm$ 5.3	76.0 $\pm$ 8.4	<u>5.68<math>\pm</math>0.18*</u>	1.00 $\pm$ 0.52
	BF	30.9 $\pm$ 5.3	62.8 $\pm$ 5.4	5.27 $\pm$ 0.15	0.58 $\pm$ 0.59
12 weeks	SF	14.1 $\pm$ 9.4	73.9 $\pm$ 11.9	6.88 $\pm$ 0.20	0.91 $\pm$ 0.80
	OSF	18.4 $\pm$ 7.0	76.1 $\pm$ 12.1	<u>5.60<math>\pm</math>0.18*</u>	1.30 $\pm$ 0.99
	BF	42.1 $\pm$ 7.1	60.4 $\pm$ 4.9	5.29 $\pm$ 0.17	1.40 $\pm$ 1.38
16 weeks	SF	1.7 $\pm$ 1.2	68.6 $\pm$ 14.0	7.09 $\pm$ 0.15	1.00 $\pm$ 0.80
	OSF	18.5 $\pm$ 5.7	67.7 $\pm$ 11.7	<u>5.60<math>\pm</math>0.20*</u>	1.30 $\pm$ 0.76
	BF	45.1 $\pm$ 9.0	59.2 $\pm$ 6.9	5.68 $\pm$ 0.24	0.89 $\pm$ 0.78

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Table 6 - relative amounts of SCFA (% of total SCFA), lactate (% of total acids), % *Bifidobacteria* between birth and 16 weeks of age.

Day / week		Acetate	Propionate	Butyrate	Sum iC4-C5	% <i>Bifidobacteria</i>	lactate
5d	SF	84.3±3.4	12.9±3.2	1.7±0.5	1.1±0.4	45±3.6	13.8±18.5
	OSF	85.8±5.1	12.0±4.7	0.5±0.5	1.7±0.7	50±8.6	8.7±13.3
	BF	89.5±1.8	7.0±1.5	1.6±0.4	2.0±0.4	54±4.1	12.5±14.0
10d	SF	70.9±2.0	21.3±2.6	4.6±1.1	3.2±0.5	65±6.0	4.3±10.8
	OSF	<u>84±2.4*</u>	13.5±2.3	<u>1.4±0.4*</u>	1.1±0.4	61±6.3	8.2±9.8
	BF	89.3±1.9	5.8±1.3	2.3±0.3	2.6±0.4	42±4.1	13.4±13.8
4w	SF	71.8±2.8	17.8±3.3	5.0±1.1	5.5±2.6	52±5.4	6.9±16.4
	OSF	77.7±2.2	15.4±2.0	5.8±2.2	1.1±0.3	71±4.5	8.4±10.7
	BF	91.0±1.8	4.3±1.2	2.6±0.6	2.1±0.4	47±5.4	19.6±17.0
8w	SF	74.6±2.9	16.4±2.0	6.1±1.2	2.9±0.7	50±6.3	3.5±9.9
	OSF	83.5±2.7	11.4±2.1	3.7±1.2	1.4±0.4	64±4.1	17.7±15.2
	BF	91.2±1.6	5.4±1.4	1.9±0.5	1.6±0.3	41±4.5	21.7±13.4
12w	SF	73.9±2.9	17.8±3.3	5.0±1.1	3.2±0.5	56±5.4	5.4±11.9
	OSF	86.5±2.1	11.2±1.8	<u>1.2±0.3*</u>	1.0±0.4	60±5.0	13.6±13.2
	BF	86.1±3.3	7.5±2.2	3.0±0.7	3.5±0.8	59±4.5	30.2±16.3
16w	SF	69.9±3.9	19.6±2.7	5.6±0.9	4.9±0.8	52±6.3	0.6±0.9
	OSF	82.2±5.3	14.3±4.9	<u>2.1±0.5*</u>	<u>1.5±0.4*</u>	69±7.7	<u>16.4±12.0*</u>
	BF	89.7±2.7	6.4±2.1	1.6±0.4	2.2±0.5	47±6.3	35.6±20.4

P<0.05 compared to SF; SF = standard formula fed, OSF = SF supplemented with

5 GOS+inulin; BF = breast fed.

The above demonstrates that in faeces of infants fed with this combination of GOS and polyfructose the pH is lowered, that more lactate is formed, and that an acid (acetate and lactate) pattern is formed which is more similar to that of breast fed babies and that

10 this effect cannot be attributed to the quantitative increase of *Bifidobacteria*.

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Example 3 - Beneficial effects of lactate and SCFA mixture on Muc-2, PGE1 and PGE2 expression.

3.1 Material and Methods

5 The effect of lactate and a SCFA mixture was analysed as described in Willemsen, LEM, Koetsier MA, van Deventer SJH, van Tol EAF (2003), Gut 52:1442-1447, with the following modifications: Lactate and a mixture of acetate/propionate/butyrate 90/5/5 was tested. For the mucus production experiments a co-culture of CCD18 and T84 cells was used, whereas for the PGE1 and PGE2 production experiments a  
10 monoculture of CCD18 cells was used.

3.2 Results

SCFA, in a mixture of 90/5/5 (acetate/propionate/butyrate), and L-lactate dose dependently induces MUC-2 expression in a co-culture of CCD18 and T84 cells as can  
15 be seen in Figure 4A. Also the concentration of PGE1 and PGE2 increases in CCD18 cells as can be seen in Figure 4B. At higher concentrations of the added organic acids the increases reaches statistically significance.

Example 4: Effect of lactate on colon contraction

20

4.1 Material and Methods

Male Wistar rats (CKP/Harlan, Wageningen/Horst, Netherlands) were housed under conditions of controlled temperature and light cycle and were provided free access to food pellets and water. Animals were anaesthetised by a mixture of N<sub>2</sub>O, O<sub>2</sub> and  
25 isofluran, the abdomen was opened and the colon was removed immediately. The tissue was placed in Krebs-Henseleit buffer pH 7.4 (composition in mM: 118.0 NaCl, 4.75 KCl, 1.18 MgSO<sub>4</sub>, 2.5 CaCl<sub>2</sub>, 10 glucose, 1.17 KH<sub>2</sub>PO<sub>4</sub> and 24.9 NaHCO<sub>3</sub>).

The colon was cut into a distal and a proximal portion and was rinsed with Krebs-Henseleit buffer while gently squeezing out faecal content. To approximate *in vivo*  
30 conditions as closely as possible, 1 cm fully intact segments were attached longitudinally to an isometric force transducer (F30 type 372, HSE, Germany) in 20 ml water-jacketed (37°C) organ baths (Schuler, HSE, Germany) containing Krebs-Henseleit buffer gassed continuously with 95% O<sub>2</sub>-5% CO<sub>2</sub>. The segments were

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gradually stretched to a resting tension of 1 g and allowed to equilibrate for 45 minutes with intermittent washings. The tensions of the segments in rest and in response to different stimuli were amplified by a transducer amplifier module (HSE, Germany) and recorded on a multi-pen recorder (Rikadenki, HSE, Germany).

- 5 The segments were incubated with 40 mM KCl for 5 minutes and the contractile responses were measured. KCl was washed out by three consecutive washes at 5 minutes intervals. The segments were then incubated with increasing concentrations up to 100 mM of acetate or sodium-L-lactate. The acid solutions were prepared freshly in distilled water. NaOH was added to acetate to obtain a neutral pH. At the end of the
- 10 incubation with a fatty acid, 40 mM KCl was added to determine whether the contractile response to KCl was influenced by the fatty acid. Before a new incubation the segments were allowed to equilibrate for 45 minutes in fresh Krebs-Henseleit buffer with intermittent washings.

- The experimental protocol consisted of two proximal and two distal sections of the
- 15 colon. For data analysis (n=3), the contraction level induced by the stimuli was defined as the tension in g after 5 minutes incubation. Data obtained from identical segments (proximal or distal) were used to calculate a mean value and each segment served as its own control sample.

## 20 4.2 Results

As can be seen from Figure 5, sodium acetate and especially sodium-L-lactate decrease the tension of tonic contractions. The relaxation effects are higher in the distal part of the colon than in the proximal part of the colon.

- Also the number of spontaneous contractions, the phasic contractions, decrease in the
- 25 proximal part of the colon upon addition of the sodium acetate and sodium-L-lactate, whereas no effects are observed in the distal part of the colon.

- In the proximal part of the colon the tonic contractions as a response to KCl, however, are comparable in the presence or absence of 25 mM sodium acetate or sodium L-lactate. At higher concentrations a significant relaxation is observed even after addition
- 30 of KCl.

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Claims

1. Use of galacto-oligosaccharides and polyfructose for the manufacture of a composition for the treatment or prevention of abdominal bloating, gas formation,  
5 abdominal pain and/or flatulence.
2. Use of galacto-oligosaccharides and polyfructose for the manufacture of a composition for the treatment or prevention of allergy, eczema or atopic diseases
- 10 3. Use of galacto-oligosaccharides and polyfructose for the manufacture of a composition for the relaxation of contractions of the colon, preferably the tonic and/or phasic contractions.
4. Use of galacto-oligosaccharides and polyfructose for the manufacture of a  
15 composition for the treatment or prevention of irritable bowel syndrome or inflammatory bowel disease.
5. Use of galacto-oligosaccharides and polyfructose for the manufacture of a composition for the increase of intestinal barrier functioning and/or mucus production  
20 of the large intestine.
6. Use according to any one of the preceding claims, wherein
- intestinal and/or faecal short chain fatty acid levels are increased,
  - intestinal and/or faecal acetate levels relative to total short chain fatty acids are  
25 increased,
  - intestinal lactate levels are increased,
  - intestinal and/or faecal pH is decreased,
  - the sum of intestinal and/or faecal butyrate, isobutyrate, valerate and/or isovalerate relative to total short chain fatty acids is decreased and/or  
30 - intestinal gas production is decreased,
- without having a significant effect on the total and/or relative number of intestinal bifidobacteria.



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7. Use according to any one of the preceding claims, wherein the amount of galacto-oligosaccharides : polyfructose has a ratio of 3:97 to 97:3, preferably 5:95 to 95:5, more preferably 90:10 to 45:55.

5 8. Use according to any one of the preceding claims , wherein the galacto-oligosaccharide is transgalacto-oligosaccharide and/or the polyfructose is inulin.

9. Use according to any one of the preceding claims, wherein the polyfructose is inulin with an average degree of polymerisation of 20 or above.

10

10. Use according any one of the preceding claims, wherein said composition is suitable for decreasing the faecal and/or intestinal pH to below pH 6, preferably to pH 5.75 or less, and wherein said composition is suitable for oral administration to an infant.

15

11. Use according to any one of the preceding claims, wherein said composition is suitable for increasing the intestinal and/or faecal lactate to above about 10 mmol/kg faeces, and wherein said composition is suitable for oral administration to an infant.

20 12. Use according to any of the preceding claims, wherein said composition is suitable for increasing the intestinal and/or faecal acetate to above about 85% of the total SCFA.

13. Use according to any of the preceding claims, wherein said composition is suitable for decreasing the sum of intestinal and/or faecal butyrate, isobutyrate, valerate and  
25 isovalerate to below 7% of total SCFA.

14. Use according to any of the preceding claims for promoting the production of short chain fatty acids at the beginning, middle and end of the colon.

30 15. Use according to any one of the preceding claims, wherein the composition is a food composition or food supplement composition, preferably an infant nutrition, more preferably an infant or follow-on formula.

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Fig 1

Pattern of SCFA formation

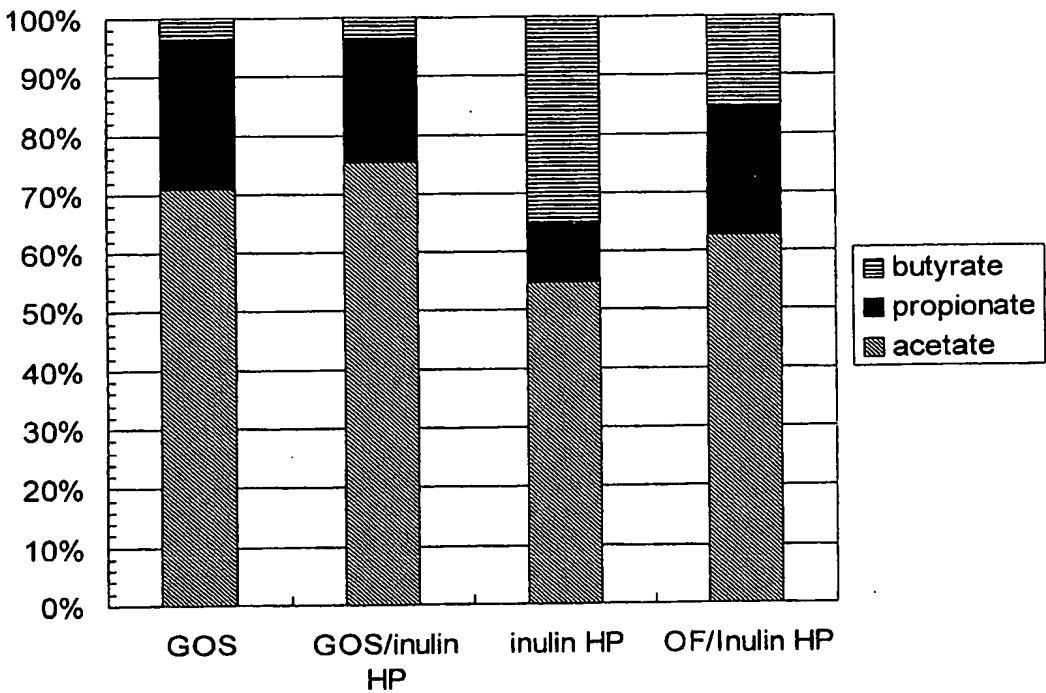


Fig 2

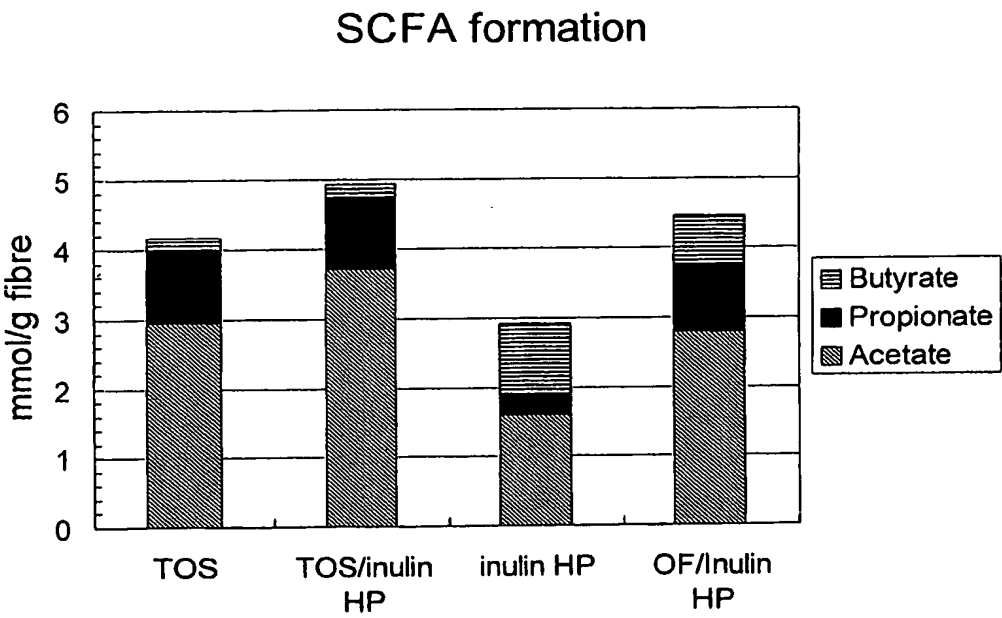


Fig 3

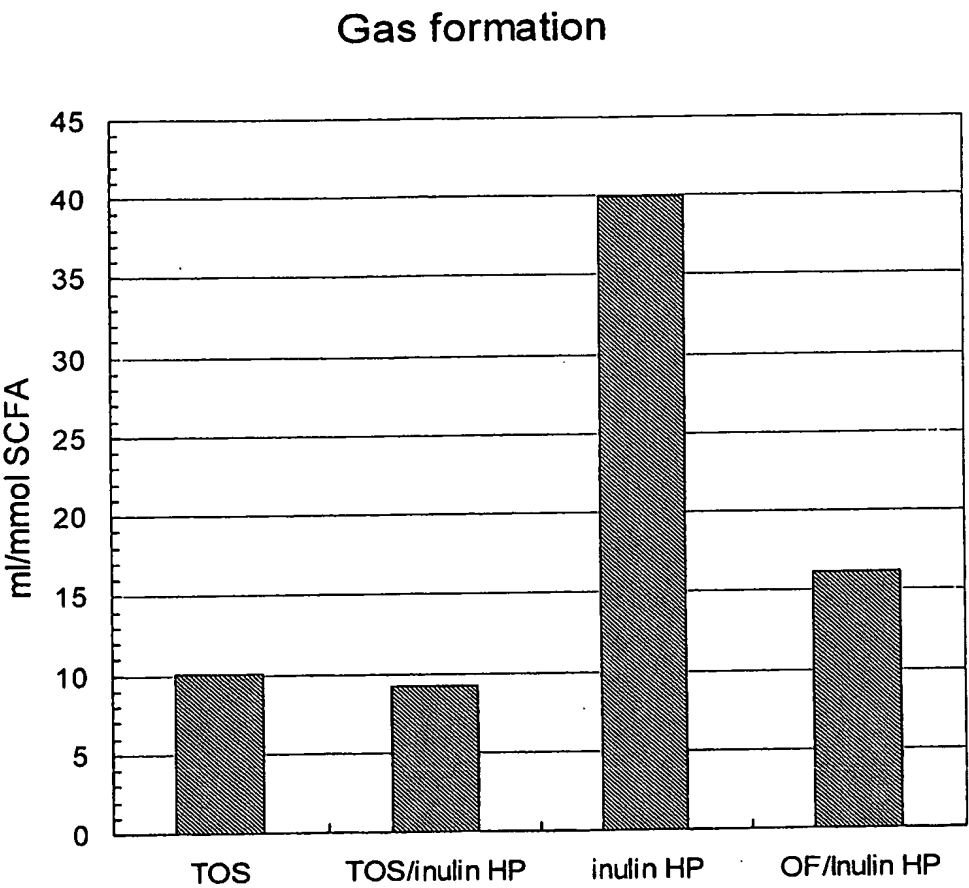
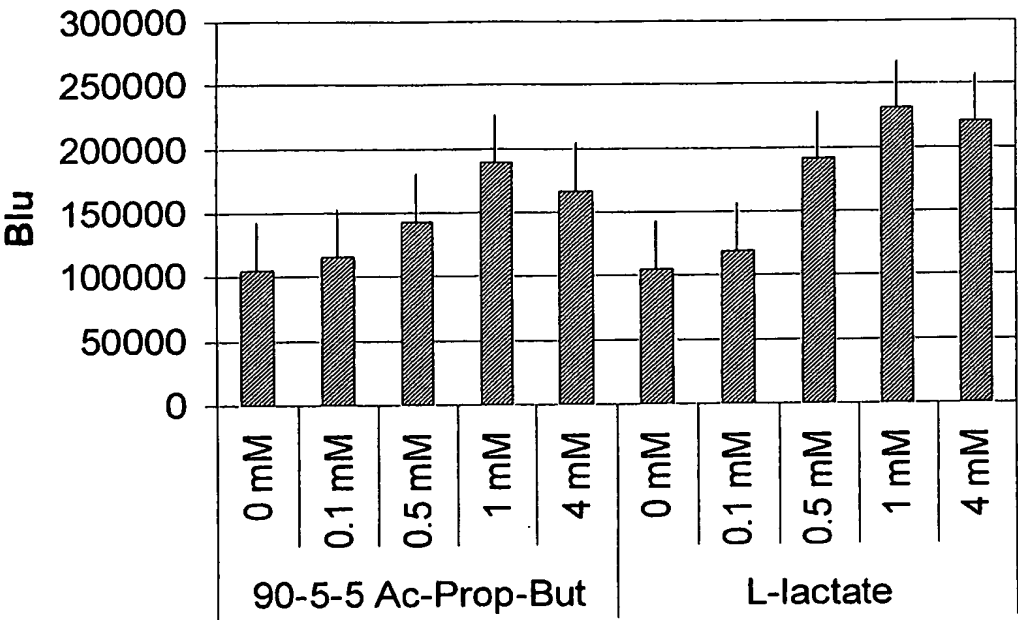


Fig 4a

A: Mucus expression



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Fig 4b

B: PGE synthesis

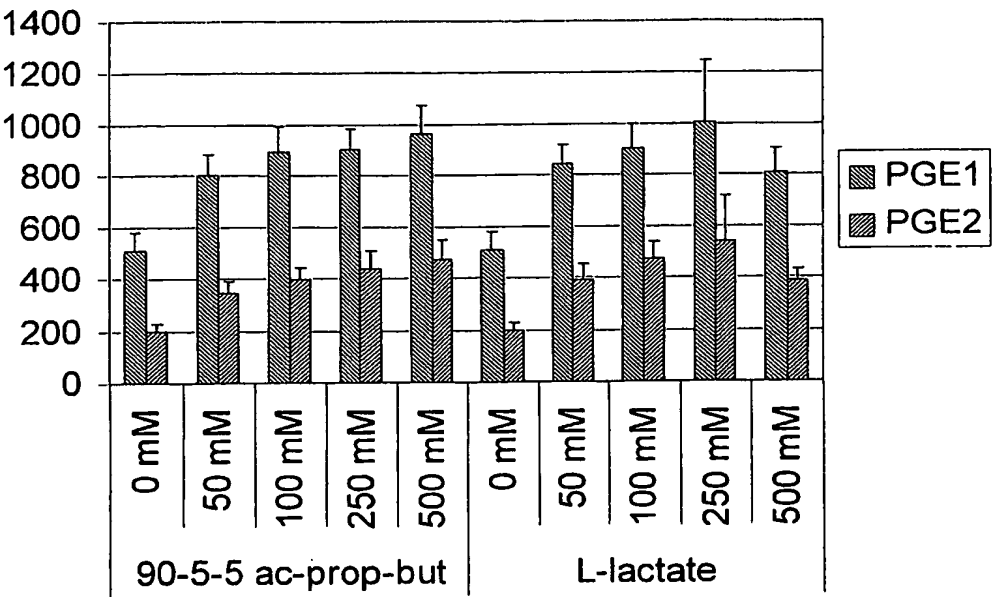
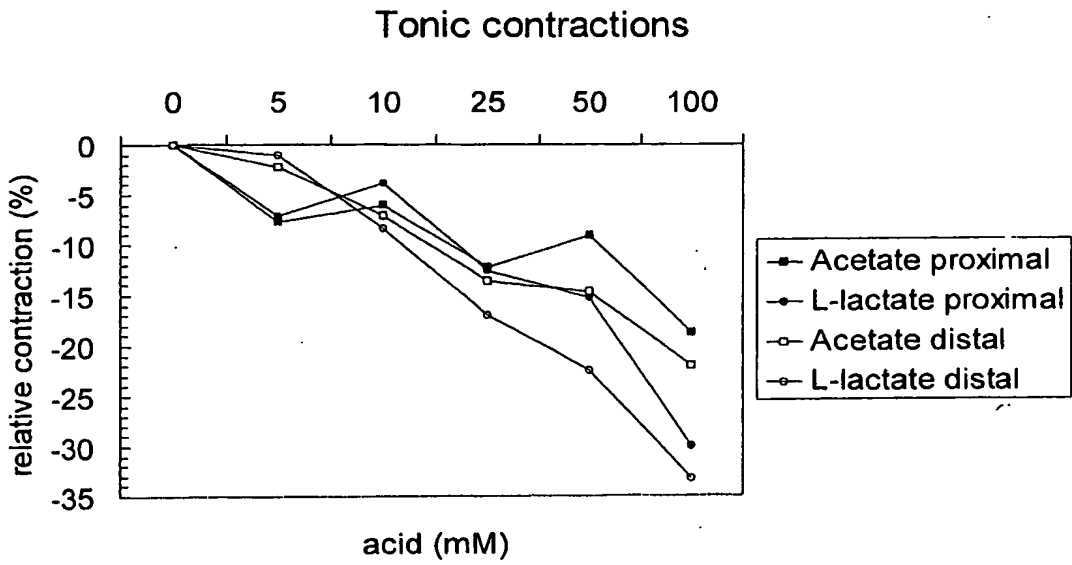


Fig 5



## INTERNATIONAL SEARCH REPORT

Application No  
PCT/NL2005/000372

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A23L1/29 A23L1/30 A61K31/01 A61K31/702 A61K31/715  
A61K31/733 A23L1/0528

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, FSTA, WPI Data, INSPEC

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>WO 2004/052121 A (NOVARTIS NUTRITION AG ; BEER MICHAEL (CH); GIBSON GLENN R (GB)) 24 June 2004 (2004-06-24) page 1, line 1 - page 2, line 12 page 3, line 11 - page 4, line 26 page 5, line 11 - page 9, line 14 page 11, line 10 - page 16, line 34 claims</p> <p style="text-align: center;">----- -/--</p>	4, 6, 8, 14, 15



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the International filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the International filing date but later than the priority date claimed

- \*T\* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the International search

5 October 2005

Date of mailing of the International search report

20/10/2005

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Popa, M



# INTERNATIONAL SEARCH REPORT

Application No  
PCT/NL2005/000372

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOEHM G., FANARO S., JELINEK J., STAHL B., MARINI A.: "Prebiotic concept for infant nutrition" ACTA PAEDIATRICA. SUPPLEMENT, vol. 91, no. 441, September 2003 (2003-09), pages 64-67, XP008033731 ISSN: 0803-5326 cited in the application the whole document	3,6-15
X	MORO G ET AL: "Dosage-Related Bifidogenic Effects of Galacto- and Fructooligosaccharides in Formula-Fed Term Infants" JOURNAL OF PEDIATRIC GASTROENTEROLOGY AND NUTRITION, RAVEN PRESS, NEW YORK, NY, US, vol. 34, no. 3, March 2002 (2002-03), pages 291-295, XP008029577 ISSN: 0277-2116 cited in the application the whole document	3,6-15
X	MORO G.E. ET AL: "Effects of a new mixture of prebiotics on faecal flora and stools in term infants" ACTA PAEDIATRICA. SUPPLEMENT, vol. 91, no. 441, September 2003 (2003-09), pages 77-79, XP008033732 ISSN: 0803-8326 cited in the application the whole document	3,6-15
X	BÖCKLER H.-M.: "Prebiotics in infant nutrition" INTERNET ARTICLE, 'Online! 8 July 2001 (2001-07-08), pages 1-5, XP002292254 Retrieved from the Internet: URL:http://www.se-neonatal.es/se-neonatal/ Oviedo2001/prebiotics.pdf> 'retrieved on 2004-08-11! the whole document	3,6-15
A	EP 1 105 002 B (NUTRICIA NV) 17 July 2002 (2002-07-17) paragraphs '0013! - '0018!, '0027! - '0033!, '0042! claims; examples	6-15

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INTERNATIONAL SEARCH REPORT		Application No PCT/NL2005/000372
C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>RIGO J ET AL: "GROWTH, WEIGHT GAIN COMPOSITION AND MINERAL ACCRETION IN TERM INFANTS FED A NEW EXPERIMENTAL FORMULA CONTAINING HYDROLYSED PROTEIN, BETA-PALMITATE AND PREBIOTICS" PEDIATRIKA, ALPE, MADRID, ES, vol. 21, no. 10, 2001, pages 387-396, XP008029568 ISSN: 0211-3465 the whole document</p> <p>-----</p>	

## INTERNATIONAL SEARCH REPORT

application No.  
PCT/NL2005/000372**Box II Observations where certain claims were found unsearchable (Continuation of Item 2 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

International Application No. PCT/NL2005 /000372

**FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 6-15 (part)

Treatment/prevention of abdominal bloating, gas formation  
and/or flatulence  
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2. claims: 1, 6-15 (part)

Treatment/prevention of abdominal pain  
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3. claims: 2, 6-15 (part)

Treatment/prevention of eczema or atypical diseases  
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4. claims: 2, 6-15 (part)

Treatment/prevention of allergy  
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5. claims: 3, 6-15

Relaxation of colon contractions  
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6. claims: 4, 6-15

Treatment/prevention of IBD/IBS  
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7. claims: 5, 6-15 (part)

Increasing of intestinal barrier functioning  
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8. claims: 5, 6-15 (part)

Increasing the mucus production of the large intestine  
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# INTERNATIONAL SEARCH REPORT

Application No  
PCT/NL2005/000372

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004052121 A	24-06-2004	AU 2003294835 A1 CA 2508693 A1 EP 1571923 A1 US 2004131659 A1	30-06-2004 24-06-2004 14-09-2005 08-07-2004
EP 1105002 B	17-07-2002	AT 220511 T AU 766429 B2 AU 5514699 A BR 9912959 A CA 2340103 A1 CN 1105506 C DE 19836339 A1 DK 1105002 T3 WO 0008948 A2 EP 1105002 A2 ES 2180321 T3 ID 29945 A JP 2002522461 T NZ 510347 A PL 346642 A1 PT 1105002 T	15-08-2002 16-10-2003 06-03-2000 08-05-2001 24-02-2000 16-04-2003 24-02-2000 11-11-2002 24-02-2000 13-06-2001 01-02-2003 25-10-2001 23-07-2002 25-07-2003 25-02-2002 31-12-2002